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13. ABSTRACT (Maximum 200 words)

Mitochondria are subcellular organelles present in cells of all tissues (except sperm) that carry out the oxidative-phosphorylation (OXPHOS) metabolic reactions that are essential for and central to cellular energy production. Abnormal mitochondrial function profoundly and adversely affects human health and performance, contributing to the development of numerous diseases including diabetes, ophthalmologic defects, deafness, neuromuscular disorders, defects in oxidative phosphorylation, and possibly cancer. Additionally, a decline in mitochondrial function is believed to be the major contributor to reduced physical and perhaps cognitive capacity during human aging. Unlike lower eukaryotes such as yeasts, cells in multicellular eukaryotes require fully-functioning mitochondria for viability. Mutations in these mitochondrial-associated genes (or damage to regulatory or structural mitochondrial proteins) can significantly impair mitochondrial function and result in a progressive reduction in energy output, significantly below that needed in body tissues. This can result in the manifestation of aging-related endpoints including reduction or loss of memory, hearing, vision, stamina, and the onset of age-related diseases including Parkinson's disease, neuromuscular defects, and cancer. Our research focus is the identification and characterization of genetic and biochemical factors associated with mitochondrial DNA (mtDNA) maintenance and the fidelity of the mtDNA polymerase.

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RESULTS

1. Generation and characterization of a *S. cerevisiae* strain bearing an exonuclease-deficient mitochondrial DNA polymerase (*mip1*exo$^-$).

(a) Introduction: The exonuclease-deficient (exo$^-$) version of the *S. cerevisiae* mitochondrial DNA polymerase gene *MIP1* was constructed by site-directed mutagenesis of a wild-type plasmid-encoded copy. This construct was used to confirm:

(i) the prediction that the presence of this mutation would increase the *arg8*^{m} (mitochondrial) mutational rate; and,

(ii) that *mip1*exo$^-$ would have an additional effect on the mitochondrial mutational rate when combined with the *pos5* mutation (please refer to Item #2, below). This work has been completed and published (Strand *et al.* 2003).

(b) Main findings: The plasmid-borne *mip1*exo$^-$ mutation, in cells carrying the wild-type *MIP1*$^+$ gene in the nucleus, increased the *arg8*^{m} mutation rate approximately 6-fold; disruption of the endogenous *MIP1*$^+$ gene enhanced this effect an additional ~1.6-fold (~10-fold above the wild-type strain). Using the *ade2* red-white colony color assay (see Ugolini and Bruschi 1996 and references therein), *pos5* increased the mitochondrial petite (white) colony frequency 4-6-fold, as did the plasmid-encoded *mip1*exo$^-$ (in a *MIP1*$^+$ cell). The petite frequency in the *pos5 MIP1*$^+$ cell carrying the plasmid-encoded *mip1*exo$^-$ was elevated 15-fold.

(c) Conclusions: This, and additional *mip1*exo$^-$ mutants (constructed but 'not shown') will be useful for additional studies involving the replication and maintenance of the mitochondrial genome, including the role of putative mitochondrial endo/exonucleases (*e.g.* Din7p; please refer to Item #3, below).

2. Cloning of the wild-type *S. cerevisiae POS5* gene, a mitochondrial mutator gene, and expression and characterization the protein product.

(a) Introduction: Using the *arg8*^{m} assay, Micheline Strand determined that disruption of the nuclear-encoded *S. cerevisiae POS5* (peroxide-sensitive) gene dramatically increased (~50-fold) the mitochondrial mutational rate. The *POS5* gene product (protein), of previously unknown function, localizes in the mitochondrion; additionally, *pos5* mutants display a slow-growth phenotype (especially on glycerol), an increased mitochondrial mutation rate, an increase in mitochondrial 'petite' colony formation, an increase in mitochondrial DNA copy number, and an acute sensitivity to copper (a soluble oxidizer). During the course off this work BLAST searches against sequences in GenBank indicated that the *POS5* gene product was likely to be a NAD+ kinase.

(b) Greg Stuart cloned the wild-type (full-length) *POS5* gene from two different *S. cerevisiae* strains, as well as two clones that deleted predicted mitochondrial target sequence peptide sequences. The full-length protein was completely insoluble, whereas the NH_2-terminus partial deletants were expressed in soluble form.

(c) This protein was subsequently demonstrated by a collaborator to have NAD/NADH kinase activity. The gene is also homologous to two recently described *S. cerevisiae* mitochondrial NAD kinases, that are distinct from this previously unknown gene function.

d) Greg Stuart recently constructed yeast strains (wild-type; *pos5* mutant) containing homomononucleotide tracts of 5 and 14 adenosines in the chromosomal *lys2* gene (Tran *et al.* 1997), to determine whether there was any effect of the *pos5* mitochondrial mutator on chromosomal mutational rates. The *pos5* mutation had no significant effect on the reversion rates of the A_5 (*lys2*-$A_5 \rightarrow LYS^+$) or the A_{14} (*lys2*-$A_{14} \rightarrow LYS^+$) runs. In contrast, *pos5* increased the *arg8*^{m} (mitochondrial) mutational rate ~50-fold, as previously mentioned.

(e) Conclusions: These findings, including the *mip1*exo$^-$ data (above), have been published (Strand *et al.* 2003). We concluded that the Pos5p NADH kinase is required for mitochondrial DNA stability, with a critical role in detoxification of reactive oxygen species. These results predict a role for NADH kinase in human mitochondrial diseases.

3. Investigation of the role of the *DIN7* gene in DNA replication and/or repair in the mitochondrion.

(a) Introduction: *DIN7* is a DNA damage-inducible gene of unknown function, the product of which localizes to the mitochondrion (Mieczkowski *et al.* 1997; Fikus *et al.* 2000; Koprowski *et al.* 2003). *Din7* mutants decrease the number of mitochondrial petite mutants caused by *dun1* mutants, another damage-inducible gene, whereas overexpression of non-mutant *DIN7* increases the petite frequency. The closest homolog to *DIN7* is *EXOI*, but Din7p cannot compensate for EXOI in mismatch repair. *DIN7* is also homologous to yeast *RAD27* (human *FEN1*), the 'flap endonuclease' involved in the processing of Okazaki fragments during DNA lagging strand replication, and the DNA repair gene XPGC, also a member of the Fen1 family of structure-specific nucleases. We postulate that Din7p is a mitochondrial flap-endonuclease, involved in mitochondrial Okazaki DNA fragment processing.

(b) We are investigating the role of the *DIN7* gene product *in vivo* in yeast mitochondria, alone and in combination with a chromosomal *mip1*exo$^-$ mutation (constructed using 'delitto perfetto' site-directed mutagenesis: see Storici *et al.* 2001, and Item #1, above).

(c) Haploid yeast strains separately containing the *mip1*exo$^-$ or *din7*Δ mutations were mated, sporulated, and the haploid spores were dissected and characterized ('tetrad analysis'). In a wild-type (*MIP1*$^+$) background, *din7*Δ mutation was found to decrease the mitochondrial petite frequency (expected), and the cells containing the *mip1*exo$^-$ *din7*Δ double mutation demonstrated a small, but significant increase in the elevated petite frequency that is observed in *mip1*exo$^-$ cells (unpublished results), indicating a small effect of a *din7* mutation on mitochondrial DNA replication in *mip1*exo$^-$ mutant cells. These preliminary data suggest that Din7p probably has a role distinct from the putative Fen1-like (Flap-endonuclease) activity, suggested by protein homology.

(e) We have also cloned the full-length *DIN7* gene, as well as two clones that delete predicted mitochondrial targeting sequences, in a histidine-tagged protein expression vector. Plans to biochemically characterize the activity of the purified Din7p protein, using synthetic (oligonucleotide) substrates, and other methods are currently being reviewed.

(f) Conclusions: The future direction of this work is currently under review, given the non-synergy of the mip1exo$^-$, din7Δ double-mutant.

4. Generation and characterization of *S. cerevisiae* mtDNA polymerase (*MIP1*) mutants analogous to those associated with the human neuromuscular disease, progressive external ophthalmoplegia (PEO).

(a) Introduction: Recently, it was shown that specific mutations in the human mitochondrial DNA polymerase gene (*POLG*) are associated with the development of progressive external ophthalmoplegia

(PEO) (Van Goethem *et al.* 2001). The Copeland group demonstrated that these mutations result in decreased replicative fidelity in human DNA polymerase γ, *in vitro* (Ponamarev *et al.* 2002).

(b) Progress: To investigate the effects of PEO mutations *in vivo*, Greg Stuart has constructed homologs of the human PEO mutations using 'delitto perfetto' site-directed mutagenesis (Storici *et al.* 2001) in the yeast mitochondrial DNA polymerase (*MIP1*) gene of *S. cerevisiae* strain YPH925 (Spencer *et al.* 1994), one of our commonly-employed 'working' laboratory strains. The PEO mutations primarily affect the exonuclease or the polymerase B domains of the (human) POLG (and the yeast *MIP1*) gene; the former set of mutations are expected to result in mutator phenotypes; the latter set of mutations will likely severely decrease polymerase activity. Preliminary data using the YPH925 strain (bearing the wild-type $MIP1^+$ gene on a plasmid to maintain the mitochondrial genome during disruption of the chromosomal *MIP1* copy) indicate an increased petite frequency; however, some of the PEO mutations are too powerful to allow the cells to be cured of the p$MIP1^+$ plasmid. Thus, it was decided to modify this approach, slightly, as follows. Although strain YPH925 has several useful properties (e.g. the *ade2* red-white colony color selection mentioned previously: see Item #1, above), the *kar1* mutation complicates mating of haploid strains by preventing nuclear fusions and thus the mixing and segregation of nuclear gene alleles. It has been decided that the PEO studies would better conducted in a yeast strain that mates and sporulates well, thus permitting tetrad analyses of different PEO mutations. *S. cerevisiae* strain E134 [*MATα* (alpha) *ade5-1 lys2-*A_{14} *his7-2 leu2-3,112 trp1-289 ura3-52*; *his7-2* is a -1 frameshift mutation creating a run of 7 adenines, that reverts primarily by +1 changes; *lys2-*A_{14} allele is a frameshift reporter containing a run of 14 adenines that via a -1 frameshift mutation] (a gift from Dr. Dmitry Gordenin, NIEHS) was subsequently selected for study. An *ade2*Δ (and $ADE5^+$) version of this strain has been constructed, permitting red-while color monitoring of mitochondrial respiratory status (as well as respiration-defective sectoring within respiration-competent colonies). This $MIP1^+$ rho^+ (containing fully functioning mitochondria) strain will be mated with an isogenic *MAT***a** strain containing the *mip1* PEO mutations, sporulated, and subjected to tetrad (and other) analyses. (Note that this approach also eliminates the requirement for a presence of a functional $MIP1^+$ gene on a plasmid during the disruptions of the chromosomal $MIP1^+$ copy, since functional mitochondria will be restored at the mating step.)

(c) Conclusions: This work is progressing satisfactorily. The determination the *in vivo* effects of each of these mutations should provide a linkage between the human epidemiological data, our *in vitro* experiments using genetic-engineered human DNA polymerase γ mutant proteins, and *in vivo* results obtained using the yeast 'PEO' mutants.

5. Generation of S. cerevisiae microarray data to characterize the effects of different types of oxidative stress at a genomic level.

(a) Introduction: Oxidative stress is caused by a variety of internal and external factors, and results in reversible or irreversible damage to mitochondrial and nuclear lipids, proteins, and DNA. Understanding the factors that control oxidative damage is important to minimizing the effects of free radicals on cellular function.

(b) Results: High quality microarray data has been obtained for all 6000 yeast genes from the following strains: wild type, sod1, sod2, pos5, wild-type grown on glycerol, pos5 grown on glycerol, wild-type shifted to glycerol, and pos5 shifted to glycerol.

(c) Conclusions: Analysis of this data is currently underway, and the analysis and follow up work is expected to take several months.